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Glutamate receptor, identification in insect muscle
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Physostigmine, inhibition of open ACh-receptor channel

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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

A variety of drugs inhibited nicotinic acetylcholine (ACh) receptor function competitively via its agonist-binding site and/or noncompetitively via its allosteric sites. Drugs acting on the latter sites included amantadine and perhydrohistrionicotoxin, which bound to the open and closed channel conformation of the receptor. Other drugs blocked only on the open channel conformation, such as physostigmine, scopolamine, gephyrotoxin, bupivacaine, while other drugs, such as meproadifen and imipramine, blocked the closed and

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Phencyclidine, inhibition of acetylcholine receptor channel
Meproadifen, inhibition of closed ACh-receptor channel
Pyrethroids, action on acetylcholine receptor
Benzodiazepine, receptor in insect muscle
Wasp venom, inhibition of glutamate receptor

20. Abstract (continued)

intermediate nonconducting channel conformation. While m-amino-PCP blocked only the closed and intermediate nonconducting conformations, m-nitro-PCP blocked the open channel conformation as well. There was excellent correlation between the potencies of a series of aliphatic alcohols in stimulating [^{3}H]perhydrohistrionicotoxin binding to the receptor's channel sites and their membrane/buffer partition coefficients. Pyrethroids acted like alcohols and both inhibited binding to the activated receptor conformation. The alcohol moiety of the pyrethroid was more important for these effects than the acidic moiety.

Several drugs enhanced receptor desensitization, such as gephyrotoxin and meproadifen. Receptor desensitization was dependent upon temperature. Although binding of agonists to the receptor was essentially temperature independent, binding to the allosteric channel sites was highly temperature dependent. Neither ATP nor several other nucleotides affected nicotinic receptor binding.

A glutamate receptor was identified in house fly muscle preparation by virtue of its specific stereoselective binding of [^{3}H]glutamate. It was different from mammalian brain receptor in its low sensitivity to ibotenate. The venom of the Philanthus wasp was collected and found to inhibit the channel of the glutamate receptor in a voltage-dependent manner. The venom was partially purified so that each active component can be identified.

A GABA/benzodiazepine receptor was identified in housefly muscle by virtue of its specific GABA-activated [^{3}H]flunitrazepam binding. Though generally similar to the mammalian GABA receptor in its drug sensitivities, there were a few differences, such as the low affinity for clonazepam and β-carboline carboxylate.

Several anticholinesterases interacted with the nicotinic receptor. The carbamates neostigmine, pyridostigmine and physostigmine were weak agonists, while physostigmine was more potent as an allosteric inhibitor of the open-channel conformation. These carbamates had no effect on rat brain GABA receptor at the same concentrations.

NEUROTRANSMITTER RECEPTORS AND THEIR IONIC CHANNELS AS TARGETS FOR DRUGS AND TOXINS

FINAL REPORT

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4. REPORT

A. STATEMENT OF THE PROBLEM STUDIED

The overall objective was to study the drug specificity of the acetylcholine (ACh), glutamate and γ-aminobutyric acid (GABA) receptors and their associated ionic channels to determine which anticholinesterases interact specifically with any of these regulatory proteins and to understand mechanisms by which these receptors regulate the selective translocation of ions across membranes. The combination of the electrophysiological approach with the biochemical one in studies of these receptor/channel systems helps us compare them and understand better their dynamic natures and determine if and which receptor/channel system acts as a primary or secondary target for a variety of drugs and toxins.

B. SUMMARY OF RESULTS

Specific Aim #1: To Identify the Various Sites on the Acetylcholine Receptor/Channel Molecule That Bind Drugs and Toxins

Our electrophysiologic studies were conducted on frog rectus abdominis, sciatic nerve sartorius muscle preparations, rat phrenic nerve diaphragm preparations and soleus muscle using vc ge clamp, noise fluctuation analysis and patch clamp. Our biochemical studies were conducted on Torpedo electric organ membranes, which contain high concentrations of nicotinic receptors, using binding of different radiolabeled probes and receptor-activated tracer flux assays. The nicotinic acetylcholine (ACh) receptor has 'receptor sites,' which bind agonists as well as competitive antagonists such as α-bungarotoxin (α-BGT), independently of the voltage across the membrane, and different 'channel sites,' which bind noncompetitive antagonists such as perhydrohistrionicotoxin (H_{12}-HTX) or phencyclidine (PCP) in a voltage-dependent manner. Drugs may bind to one or both of these kinds of sites and affect receptor function in different ways.

Mechanisms of Interaction of Agonists

Agonists differ in their potency in activating the ACh-receptor. For example, the lifetime of the channel opened by ACh in frog sartorius muscle is 4.4 msec at -90 mV and 10°C, but 1.68 sec for (±)-muscarone and 2.33 msec for tetramethylammonium (Spivak et al., 1983). We investigated the structure-activity relationships of a series of semirigid nicotinic agonists [anatoxin-a (-)-ferruginine methiodide, arecoline methiodide, (-)-cytisine and (±)-muscarone] (Spivak et al., 1983). Natural (+)-anatoxin-a was the most active followed by arecoline methiodide. A correlation between nicotinic potency and steric requirements indicated a central role for the positively charged groups. Channel conductance and the voltage sensitivity of channel lifetime varied among agonists, but the average charge traversing the membrane through a single open channel did not correlate with potency. Thus, it was concluded that the dominant component of potency is the frequency of channel opening.

Mechanisms of Receptor Inhibition

Blockade of receptor function occurs through one or more of three general mechanisms:

(1) Competitive antagonism: The antagonist binds to the 'receptor site' thus preventing binding of ACh and activation of the receptor. The effect of the antagonist is concentration dependent and is manifested as a reduction in peak amplitude of endplate current (epc) and miniature endplate current (mepc) without affecting channel lifetime or conductance. A competitive antagonist is detected as well by its inhibition of [^{3}H]ACh binding or [^{125}I]α-BGT to the receptor sites.

(2) Noncompetitive antagonism: The antagonist binds to 'channel sites,' causes nonlinearity in the current-voltage relationship and/or modulates the time course of epc (τ_{epc}) and channel lifetime. Thus, ACh still binds to the receptor sites, but the channel fails to open or is physically occluded by the drug once it has already opened. Binding to the channel sites is affected by the voltage across the membrane of which the AChR is a part and can be detected by inhibition of [^{3}H]H_{12}-HTX binding to the channel sites without inhibiting (and possibly potentiating) binding of [^{3}H]ACh.

(3) Receptor desensitization: The receptor becomes refractory to opening its channel, as a result of prolonged exposure to the agonist or exposure to a high concentration of it, or by binding of an antagonist to the 'channel sites,' which induces a conformational state of the receptor molecule, which has a high affinity for ACh. Desensitization encompasses at least two kinetically identifiable states (Sakmann et al., 1980; Feltz and Trautmann, 1982; Hess et al., 1982; El-Fakahany et al., 1982).

A great deal of evidence suggests that there are multiple channel sites on the ACh-receptor: Binding of [^{3}H]H_{12}-HTX or [^{3}H]PCP to the channel sites is noncompetitively inhibited with imipramine, and [^{3}H]imipramine binding is inhibited noncompetitively with histrionicotoxin (HTX) (Shaker et al., 1982). The effects on the linearity of the current-voltage relationship of the epc are dissociated from those on the time course of epc as detailed below. Thus, certain drugs such as antidepressants cause nonlinearity, but do not affect τ_{epc} (Schofield et al., 1981), while drugs such as H_{12}-HTX cause both. Certain drugs (e.g., tetraethylammonium (TEA) and atropine methyl bromide) affect epc characteristics when applied only externally to the endplate, while PCP methiodide and piperocaine methiodide act when applied internally or externally causing nonlinearity of the peak amplitude of mepc and shortening of channel lifetime (Aguayo et al., 1981).

Action of drugs on ACh-receptor function is quite complex since many drugs bind to both receptor and channel sites, activate as well as inhibit receptor function, or are inhibitory at both sites. Examples are depolarizing blockers, whose mechanism of receptor blockade we studied. We utilized binding of the allosteric probe [^{3}H]H_{12}-HTX to the channel sites as an index for receptor conformation, since its binding is very slow to the resting receptor (taking over 1 h to saturate all sites) but is much faster to the agonist-activated receptor (Fig. 1) because of increased affinity and is intermediate to the desensitized receptor (Eldefrawi et al., 1980; El-Fakahany et al., 1982). We found that certain depolarizing blockers activated the receptor by binding to the 'receptor sites' and inhibited it by binding to the 'channel sites' as shown for decamethonium biochemically (Eldefrawi et al., 1982) and electrophysiologically (Adams & Sakmann, 1978). Another depolarizing blocker, succinylcholine, did not bind to the channel sites but inhibited the receptor, possibly by inducing its desensitization. Other examples are anticholinesterases, which are described under specific aim #6.

An example of a receptor antagonist which binds to both the 'receptor' and 'channel' sites is d-tubocurarine as shown by its inhibition of [^{3}H]ACh binding to the receptor sites and [^{3}H]H_{12}-HTX to the channel sites (Shaker et al., 1982). Its channel effect is reflected in depressing peak epc amplitude in a voltage-dependent manner, departure from linearity in the current-voltage relationship and changes in the time course of the epc. We found that it bound to the open receptor channel conformation as previously reported (Katz & Miledi, 1978; Colquhoun et al., 1979) for it also had higher potency on mepc and [^{3}H]H_{12}-HTX binding at 30°C than 20°C. In addition we found that it also bound to the closed channel, since it inhibited [^{3}H]H_{12}-HTX binding to the α-BGT-inhibited receptors (Shaker et al., 1982).

The mechanism of action of noncompetitive antagonists occurs via binding to the channel sites in a resting closed-channel conformation, activated but nonconducting conformation and/or open channel conformation. In the case of the latter, the more the activation of the receptor the more potent is the

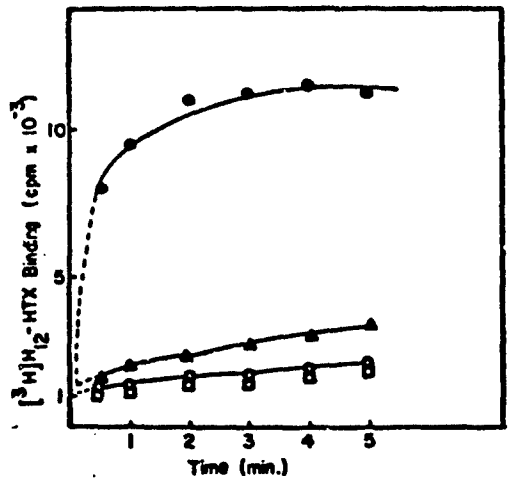

Fig. 1. Effect of receptor ligands on the kinetics of binding of [^{3}H]H_{12}-HTX to Torpedo membranes. Each symbol is the mean of three experiments, and the standard deviation is <10%. Binding of [^{3}H]H_{12}-HTX (2 nM) alone (o) and with the simultaneous addition of carbamylcholine (20 μM) (•), carbamylcholine (20 μM) and d-tubocurarine (100 μM) (Δ) to untreated membranes as well as to membranes pretreated with 10 μM α-BGT for 60 min before the addition of [^{3}H]H_{12}-HTX (2 nM) and carbamylcholine (20 μM) (□). (From Eldefrawi et al., 1982)

blockade. Several drugs were found to interact with the open as well as closed channel conformation of the ACh-receptor. Examples are H_{12}-HTX (Albuquerque et al., 1974), PCP (Albuquerque et al., 1980), amantadine (Tsai et al., 1978) and its N-alkyl analogs (Warnick et al., 1982b). Other drugs interacted with either the open or closed channel conformations as detailed below.

Interaction with the Open Channel Conformation

Binding of a noncompetitive antagonist to the open channel conformation is detected by a change in the time course of epc and channel lifetime and by nonlinearity in the epc voltage-current relationship. In addition, such a drug would inhibit [^{3}H]H_{12}-HTX binding to the channel sites if the receptor sites were not preinhibited with α-BGT, a treatment that retains the receptor's channel in a closed conformation.

We found that amantadine, PCP and H_{12}-HTX bound to the receptor's open- as well as closed-channel conformations. Various histrionicotoxins differed in their potencies in inhibiting [^{3}H]H_{12}-HTX binding to the channel sites with a high correlation coefficient (0.96) (Fig. 2) with their potencies in reducing peak amplitude at -90 mV. They had diverse effects on epc (Albuquerque & Spivak, 1984). H_{12}-HTX decreased both peak epc amplitude and τ, two effects which appear to be independent because of the following findings (Spivak & Albuquerque, 1982): (A) The time course for onset of both phenomena are different, with maximal depression of τ obtained much faster (Fig. 3). (B) Peak amplitude, but not τ, in presence of histrionicotoxins shows time and voltage dependence that generates hysteresis in peak epc amplitude vs voltage plots. (C) Repetitive stimulation progressively reduces peak amplitude but not τ. The dichotomy in their actions may reflect two different binding sites, or more likely binding to a single

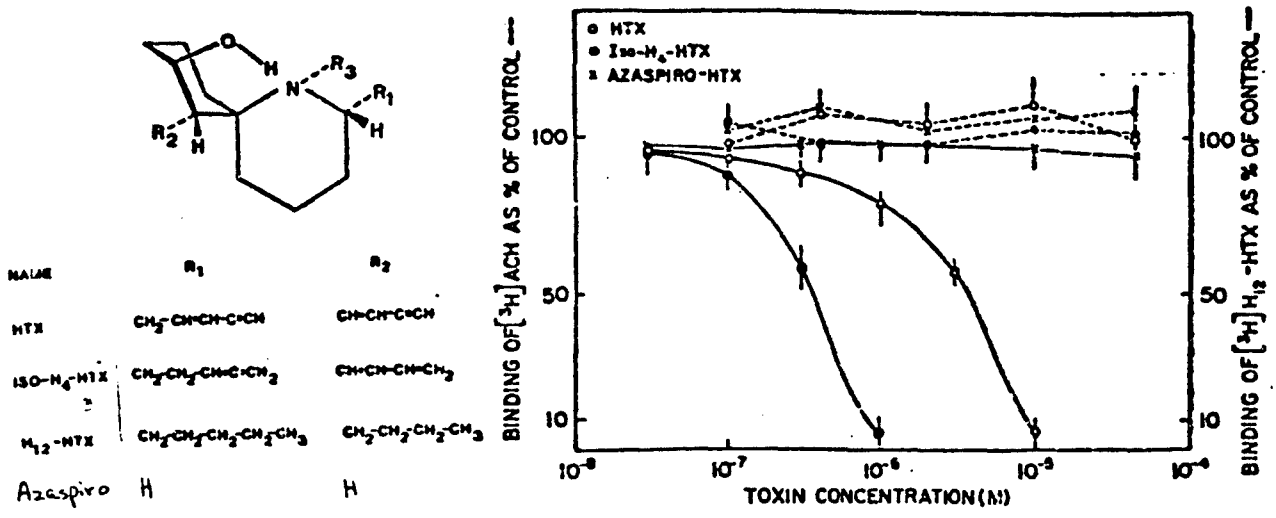

Fig. 2. The effect of HTX and analogs on the binding of [^{3}H]H_{12}-HTX (0.01 μM) and [^{3}H]ACh (1 μM) to Torpedo electric organ membranes. (From Eldefrawi & Eldefrawi, 1979)

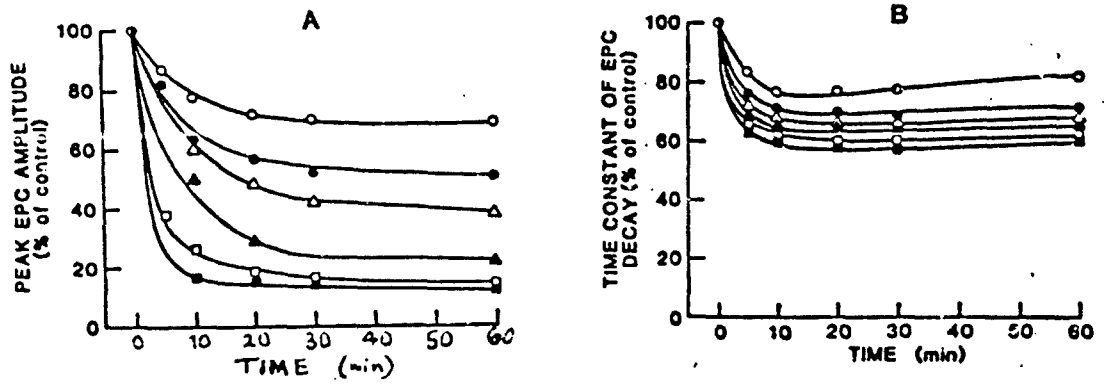

Fig. 3. Epc peak amplitudes (A) and decay time constants (B) shown as functions of time after treating a muscle with various concentrations of H_{12}-HTX. The concentrations of H_{12}-HTX used were 2 (o), 5 (•), 10 (Δ), 20 (▲), 30 (□), and 40 (■) μM. Each symbol represents the mean of 12-15 fibers from 3-5 muscles. (From Spivak & Albuquerque, 1982)

site in two different receptor conformations, especially since two affinities for [^{3}H]H_{12}-HTX are detectable: a lower affinity to the resting receptor and a higher one to the agonist-activated receptor (Aronstam et al., 1981). Possibly one conformation alters τ and the other immobilizes the receptor with a closed channel.

Removal of the two side chains of H_{12}-HTX in four N-benzylazaspiro analogs restricted their molecular interactions to the receptor's open channel conformation. Like H_{12}-HTX, they caused voltage-dependent depression of peak epc and induced nonlinearity in the voltage-current relationship, but unlike H_{12}-HTX they did not cause hysteresis or time dependency in the relationship

(Maleque et al., 1984). These effects, and the linearity of the reciprocal of the time constant of decay with increasing analog concentration (Fig. 4), suggested that the analogs interacted with the ACh-receptor only in the open channel conformation. The similar potencies of (-) and (+) H_{12}-HTX (Spivak et al., 1982) and the greatly reduced potency of azaspiro-HTX, in blocking epc amplitude and [^{3}H]H_{12}-HTX binding (Eldefrawi & Eldefrawi, 1979), supports the view that hydrophobicity plays a large role in noncompetitive blockade of the ACh-receptor. This was also demonstrated by the blockade of the channel with amine-substituted bicyclo-octane analogs of amantadine, but not by the carboxy-substituted ones (Warnick et al., 1982c).

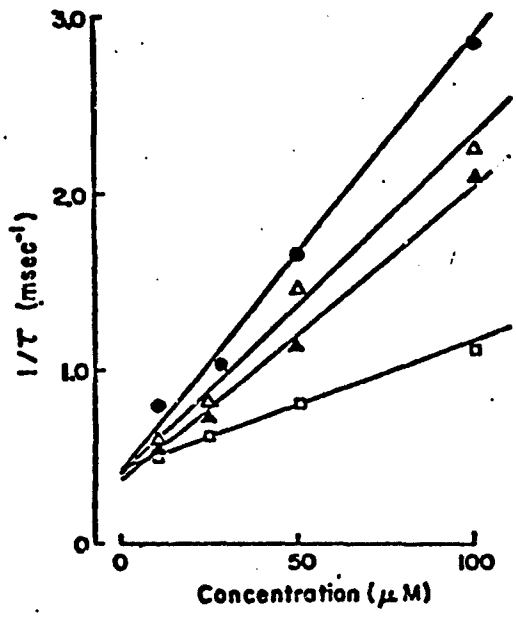

Fig. 4. The graph shows a plot of $1/\tau$ obtained from EPC decays vs. concentrations of analogs 1 (●), 2 (Δ), 3 (□) and 4 (▲) at -90 mV. The points are the mean of 8 to 32 fibers from 5 to 12 muscles. Note the linear relationship between $1/\tau$ vs. concentrations in presence of all the analogs. (From Maleque et al., 1984)

Other drugs were also suggested to interact only with the nicotinic receptor's open channel conformation. One is the local anesthetic bupivacaine, which decreased τ_{epc} and channel lifetime, but the endplate current-voltage relationship remained linear and the reciprocal of τ_{epc} with concentration was linear (Ikeda et al., 1984; Aracava et al., 1984). Another drug is the tricyclic alkaloid gephyrotoxin that was isolated from the skin of the same frogs as H_{12}-HTX. It had similar voltage-independent actions on epc and mepc (Fig. 5), and the shortening of τ_{epc} did not approach a limiting value (Souccar et al., 1984a). In addition, gephyrotoxin binding to the channel sites increased the receptor's affinity for carbamylcholine, suggesting that gephyrotoxin enhanced receptor desensitization (Souccar et al., 1984b).

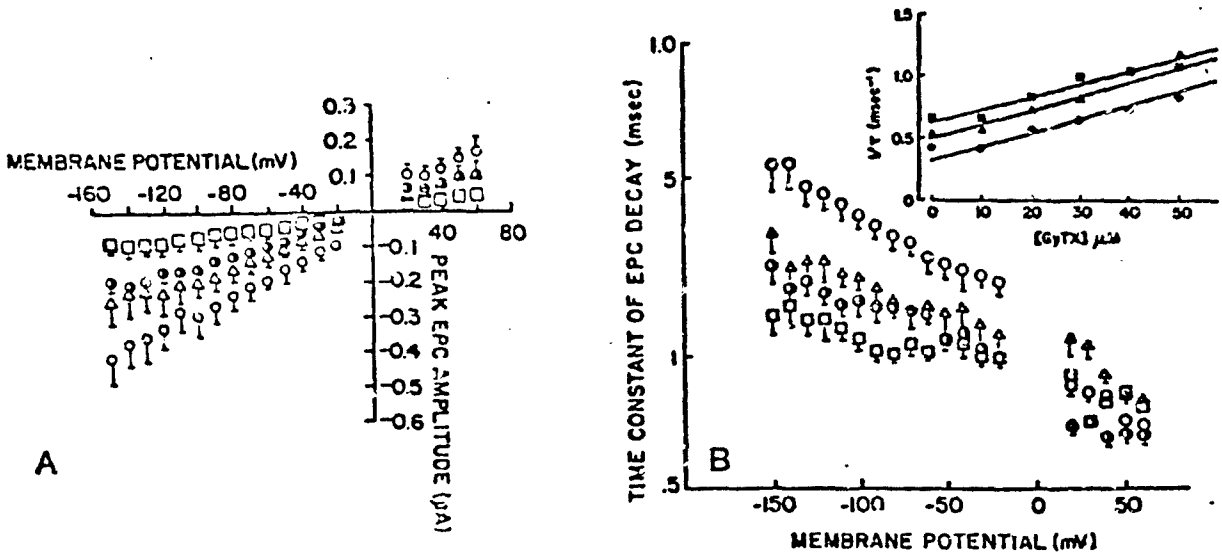

Fig. 5. Current-voltage relationship of the epc peak amplitude (A) and semilogarithmic plot of τ_{epc} against voltage (B) under control conditions (o) and after 30-min exposure of the sartorius muscle to various gephyrotoxin concentrations: 20 μM (Δ), 30 μM (o), and 40 μM (□). The I-V relationships can be considered linear for all concentrations shown. Inset in B shows the inverse of the decay time constant ($1/\tau_{epc}$) against gephyrotoxin concentration at clamp potentials of -150 mV (●), -90 mV (▲), and -50 mV (■). The relationship is linear for all voltages. Each point represents the mean ± standard error of the mean of 18 junctional regions in 3 glycerol-treated muscles for each concentration. (From Souccar et al., 1984a)

Blockade of the Closed Channel Conformation

Interaction of a drug with the closed channel conformation is detected by its voltage-dependent inhibition of peak epc amplitude and nonlinear epc current-voltage relationship, without changing τ_{epc} or single channel lifetime, as well as by its inhibition of [^{3}H]H_{12}-HTX binding to the channel site of the ACh-receptor whose receptor sites are inhibited with α-BGT. Examples of drugs that interact only with the closed channel conformation are imipramine and other phenothiazines (Table 1), which bind to a channel site that is different from the ones that bind H_{12}-HTX or PCP as shown by their noncompetitive inhibition with imipramine (Shaker et al., 1981) (Fig. 6). In order to cause nonlinearity in the current-voltage relationship of epc, the drug may also be binding to a channel site in the ACh-activated, though nonconducting, conformation since binding to the open channel conformation would affect τ_{epc}. Since some of the actions of phenothiazines appear to be agonist independent, it supports the notion that the nonconducting ACh-receptor conformation is distinct from the desensitized one (Schofield et al., 1981).

The local anesthetic meproadifen [2-(diethylmethylaminoethyl)-2,2-diphenylvalerate iodide] acted similarly, and in addition caused hysteresis loops in the endplate current-voltage relationship that were time dependent. It had no effect on τ_{epc} (Fig. 7) or single channel lifetime (Maleque et al.,

le 1. Summary of the ACh-receptor conformation with which inhibitors of the ionic nnel of the ACh-receptor drug intera

Open	Closed	Intermediate Nonconducting
, H_{12}-HTX	HTX, H_{12}-HTX	HTX, H_{12}-HTX
entyl-HTX		
-Methiodide		PCE
itro-PCP	m-Nitro-PCP	m-Nitro-PCP
	m-Amino-PCP	m-Amino-PCP
ntadine & analogs	Amantadine & analogs	
raethylammonium	Tetraethylammonium	
hyrotoxin		
erocaine	Piperocaine	
nidine	Quinidine	
trexone, naloxone	Naltrexone, naloxone	
	Meproadifen	Meproadifen
	Imipramine, desimipramine	imipramine, desimipramine
	Nortriptyline, amitriptyline	Nortriptyline, amitriptyline
allorphan		
opine, scopolamine, QNB		
amethonium	Decamethonium	
Tubocurarine	d-Tubocurarine	
stigmine, pyridostigmine		
sostigmine	Physostigmine	

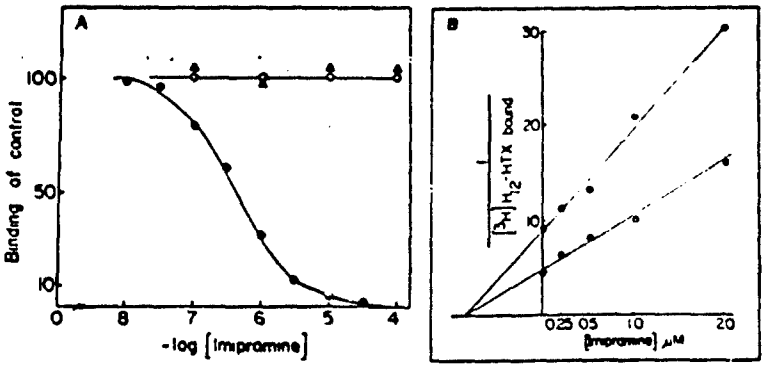

Fig. 6. Effect of imipramine on the binding of receptor and ionic channel ligands to Torpedo membranes. Each symbol is the mean of three experiments; standard deviations were less than 10%. (A) Dose-response curve of the effect of various concentrations of imipramine (10 nM - 100 μM) on the binding of [^{3}H]ACh (0.5 μM) (o), [^{3}H]α-BGT (1 nM) (Δ), and [^{3}H]H_{12}-HTX (2 nM) (●). (B) Dixon plot of the effect of imipramine (0.25 to 2 μM) on the binding of 2 nM (●) and 4 nM (o) [^{3}H]H_{12}-HTX. (From Eldefrawi et al., 1981)

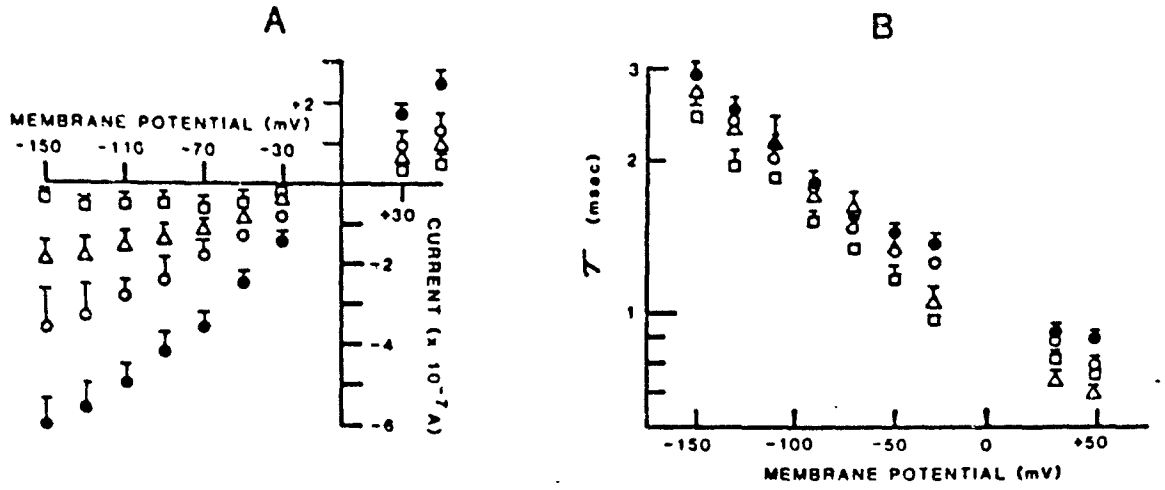

Fig. 7. Effect of meproadifen on peak epc amplitude and τ_{epc}. (A) The relationship between peak amplitude of the epc and the membrane potential is shown under control conditions (●) and after a 30- to 60-min exposure to meproadifen, 2.0 μM (o), 5.0 μM (Δ), and 10.0 μM (□). Each point represents the mean ± standard error of the mean from 12 to 46 surface fibers from at least 5 muscles. (B) The relationship between the logarithm of the τ_{epc} and membrane potential under control conditions and in the presence of meproadifen. Symbols same as in A. (From Maleque et al., 1982)

(1982). In addition, meproadifen potentiated receptor desensitization as detected by endplate potential measurements induced by iontophoretically applied ACh (Maleque et al., 1982) and by increasing the receptor's affinity for ACh (Krodel et al., 1979). Therefore, potentiation of receptor desensitization may result from interaction of the channel drug with the closed channel conformation as occurs with meproadifen, as well as the open channel conformation as occurs with gephyrotoxin (Souccar et al., 1984b).

Interactions of Alcohols and Pyrethroids with the Nicotinic ACh-receptor

Alcohols appear to interact with the ionic channel of the ACh-receptor since they modulated channel lifetime: short aliphatic alcohols (ethanol to heptanol) increased channel lifetime (Gage et al., 1975), but octanol shortened it (Gage et al., 1974). Like most noncompetitive inhibitors, alcohols stabilized a receptor conformation that had a high affinity for agonists and facilitated agonist-induced desensitization (Young & Sigman, 1981). However, we found that they differed from agonists and antagonists in their action in several of their effects: At low concentrations, alcohols and 1-naphthol acted like agonists in increasing the initial rate of $[^3H]H_{12}$-HTX binding to the channel sites and increasing the receptor's affinity for agonists, but unlike agonists they did not open channels or inhibit [^{3}H]ACh binding, nor was their potentiation of $[^3H]H_{12}$-HTX binding inhibited with pretreatment of the receptor with α-BGT (El-Fakahany et al., 1983). On the other hand, octanol, which was shown to act like an antagonist in shortening channel lifetime, did not inhibit [^{3}H]ACh or $[^3H]H_{12}$-HTX binding (El-Fakahany et al., 1983). There was excellent correlation between the potencies of the alcohols in stimulating

[^{3}H]H_{12}-HTX binding, and their membrane/buffer partition coefficients. However, interaction of the alcohol with the lipid could not by itself explain its action on the receptor since acetylation of 1-naphthol, which increased its hydrophobicity, reduced dramatically its effect on [^{3}H]H_{12}-HTX binding. Thus, the action of alcohols was suggested to be possibly due to binding to a third kind of site at the domains of the proteins embedded in the lipid bilayer.

Another group of compounds that acted like alcohols on the nicotinic ACh-receptor is pyrethroid insecticides, whose main molecular target is the axonal Na^+ channel (Narahashi, 1980). Pyrethroids had little or no effect on [^{3}H]ACh binding, and a few at high concentrations inhibited [^{3}H]H_{12}-HTX binding noncompetitively to the resting receptor state (i.e., in absence of carbamylcholine), but all at lower concentrations inhibited it to the activated state (i.e., in presence of carbamylcholine). Again, like alcohols, low concentration of the pyrethroid allethrin stimulated [^{3}H]H_{12}-HTX binding, while high concentrations inhibited it (Abbassy et al., 1982).

The pyrethroids tested could be divided into two types based on their potencies and speed of inhibition of [^{3}H]H_{12}-HTX binding as well as their structure. Pyrethrins, allethrin, bioallethrin, tetramethrin, resmethrin, esbiol and kadethrin (Type I) were more potent and acted rapidly, with their highest level of inhibition reached in <30 sec. On the other hand, inhibition of [^{3}H]H_{12}-HTX binding by permethrin, fluvalinate, cypermethrin, and fenvalerate (Type II) increased with time such that in the case of fluvalinate it was only 10% after 30 sec and 75% after 1 h. Another difference between the two types was that inhibition of [^{3}H]H_{12}-HTX binding by Type I pyrethroids increased greatly with receptor activation, while that by Type II compounds was either unaffected (e.g., permethrin, cypermethrin, and fluvalinate) or affected to lesser degrees (e.g., fenvalerate and BAY FCR 1272) (Table 2).

Table 2. The effect of pyrethroids on the binding of [^{3}H]ACh (0.1 μM) and [^{3}H]H_{12}-HTX (2 nM) to ACh-receptors of Torpedo electric organ (From Abbassy et al., 1983)

Pyrethroids (10^{-4} M)	Binding of radioactive ligands (% of control)[a]		
	[^{3}H]H_{12}-HTX		
	Unstimulated	Stimulated[b]	[^{3}H]ACh
Type I			
Allethrin	53 ± 2	4 ± 2	102 ± 8
Bioallethrin	68 ± 2	11 ± 6	113 ± 10
Natural pyrethrins	102 ± 1	12 ± 2	94 ± 7
Tetramethrin	110 ± 6	38 ± 9	100 ± 2
Resmethrin	106 ± 4	48 ± 2	114 ± 3
Type II			
Permethrin	84 ± 3	78 ± 8	98 ± 8
Cypermethrin	101 ± 6	102 ± 2	88 ± 8
Fenvalerate	112 ± 9	75 ± 6	87 ± 4
Fluvalinate	88 ± 3	77 ± 6	92 ± 4
BAY FCR 1272	112 ± 4	71 ± 4	98 ± 2

[a]Values are presented as means ± SD of three experiments.
[b]In presence of 100 μM carbamylcholine.

The alcohol moiety of the pyrethroid was more important than the acidic moiety for its action on the nicotinic ACh-receptors. Esters of cyclopentenyl (allethrin, bioallethrin, esbiol, RU 19 177 and RU 40 246), 5-benzyl-3-furylmethyl (kadethrin and resmethrin) and tetrahydrophthalimidomethyl alcohol (tetramethrin) belong to Type I compounds. On the other hand, Type II compounds are esters of α-cyano-3-phenoxybenzyl alcohol. The division of pyrethroids into two types based on their action on the nicotinic ACh-receptor is similar generally to their division based on action on the cercal sensory nerves of the cockroach recorded in vivo and in vitro and on the toxicity symptoms produced in insects (Gammon et al., 1981).

Interactions of PCP with the Nicotinic Receptor and Other Molecular Targets

We had discovered that PCP, the general anesthetic used in veterinary medicine, and a major drug of abuse that causes schizophrenia-like behavior, interacts with the channel sites of the nicotinic ACh-receptor (Albuquerque et al., 1980). Binding of [^{3}H]PCP and [^{3}H]H_{12}-HTX to the channel sites was similar in their drug sensitivities, dependence on receptor conformation and potentiation by agonists, but binding of [^{3}H]PCP was less sensitive to changes in temperature (Eldefrawi et al., 1980, 1982). Because of the commercial availability of tritiated PCP, it became a popular label for these channel sites.

Not only did PCP inhibit nicotinic ACh-receptor function, but it also blocked K^+ conductances in muscle (Albuquerque et al., 1983) and muscarinic receptors (Aronstam et al., 1980). It also bound to a site in smooth muscle membranes with higher affinity than its binding to muscarinic receptors (El-Fakahany et al., 1984). It was of interest to determine if any or all of these molecular targets for PCP were involved in its behavior-modifying effects. Therefore, we compared the potencies of several PCP analogs in inhibiting [^{3}H]PCP binding to these targets as well as 'PCP-receptors' in mammalian brain. There was poor correlation between binding to the nicotinic or muscarinic ACh-receptor and behavior effects (Eldefrawi et al., 1982). However, the behaviorally active m-amino-PCP blocked K^+ conductances, while the behaviorally inactive PCC and m-nitro-PCP did not (Albuquerque et al., 1983). It led to the suggestion that inhibition of K^+ conductance and the resulting increase of transmitter release may be related to the behavioral changes caused by PCP.

Because PCP inhibited the nicotinic receptor's ionic channel, we were interested in determining if it also affects GABA or glutamate receptor channels. Since invertebrate skeletal muscles are controlled by these receptors, we studied binding of [^{3}H]PCP to crayfish muscle. We detected high affinity binding, which was insensitive to GABA or glutamate, suggesting that [^{3}H]PCP was not binding to these receptors or their channels (Eldefrawi et al., 1982; El-Fakahany et al., 1984). Although this binding was very sensitive to Ca^{2+} channel antagonists, similar to [^{3}H]PCP binding to the nicotinic receptor (Eldefrawi et al., 1983), PCP did not inhibit the Ca^{2+} current in the muscle action potential. However, the slight prolongation of the falling phase of the action potential suggested that PCP may inhibit a K^+ channel.

Specific Aim #2: To Define Some of the Molecular Properties of the ACh-Receptor/Channel Molecule

Desensitization of the nicotinic ACh-receptor of Torpedo electric organs was studied by monitoring activated [^{3}H]H_{12}-HTX binding to the receptor's channel sites (El-Fakahany et al., 1982). Receptor desensitization was produced by incubating Torpedo membranes, which had been pretreated with diisopropylfluorophosphate, with ACh and was quenched by the addition of ACh-esterase. Desensitization caused concentration- and time-dependent decrease in the binding of [^{3}H]H_{12}-HTX to carbamylcholine-activated receptors and an increase in the apparent affinity of carbamylcholine to the receptor. d-Tubocurarine and concanavalin A antagonized the desensitizing effects of ACh. The kinetics of this desensitization showed an extremely rapid temperature- and concentration-dependent component which was complete by the earliest measurement (i.e., 6 sec) and a much slower component ($T_{\frac{1}{2}}$ = 55 sec) which reached a plateau after 120 sec. The rate of this slow component was temperature and concentration independent, but independent of the ACh concentration used for desensitization. It also showed biphasic kinetics: half the receptors recovered fast ($T_{\frac{1}{2}}$ = 36 sec), whereas the other was slow ($T_{\frac{1}{2}}$ = 180 sec).

Since receptor desensitization by the neurotransmitter was temperature dependent, we undertook a study of the effect of temperature on ligand binding to shed more light on the molecular events involved. We found that binding of ACh to the receptor sites was temperature insensitive (Fig. 8A), but the ACh-induced activation of [^{3}H]H_{12}-HTX binding was extremely temperature dependent (Fig. 8B). This temperature dependency was found with the four agonists studied. The Hill slope value of the binding of ACh to its receptor was 1.6, suggesting two interacting receptor sites. However, the Hill slope values of ACh-stimulated [^{3}H]H_{12}-HTX binding, which is also temperature dependent, increased with temperature. At 37°C the Hill slope was 2.2. Furthermore, suberyldicholine, which is more potent than ACh in inducing both receptor activation and desensitization had a Hill slope above 3 at 37°C (Table 3). Since

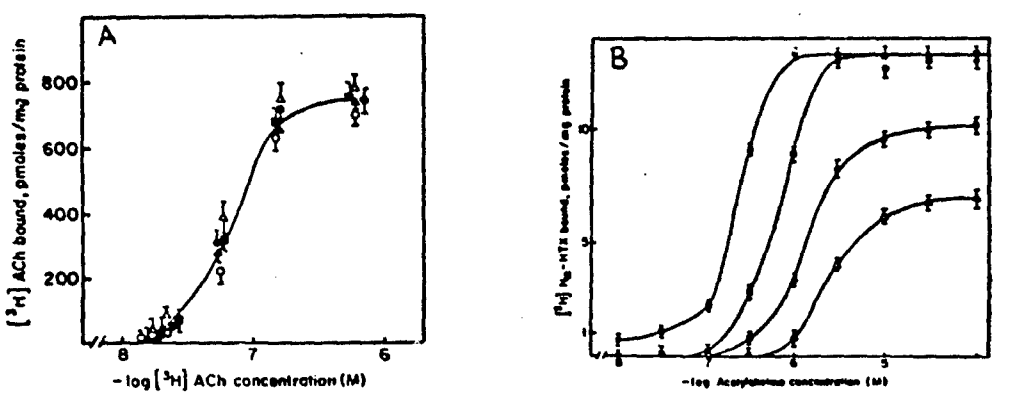

Fig. 8. Effects of temperature on binding of [^{3}H]ACh (A) and on ACh stimulation of [^{3}H]H_{12}-HTX (2 nM) binding to Torpedo membranes. Binding was measured at the following temperatures (°C): 2, Δ; 10, o; 23, ■; 32, ▲ and 37, ●, by equilibrium dialysis in A and at 30 sec by filtration in B. Symbols and bars are means and standard deviations of three experiments.

Table 3. The effect of temperature on the Hill slopes of the dose-response relationship of agonist-stimulated binding of 2 nM [^{3}H]H_{12}-HTX measured at 30 sec

Agonist	Hill slopes at different temperatures			
	2°	10°	23°	37°
Acetylcholine	0.7	1.0	1.4	2.2
Suberyldicholine	1.5	2.2	2.8	3.2
Carbamylcholine	---	---	1.5	1.4
Succinylcholine	---	---	1.2	1.1

receptor desensitization is higher at higher temperatures, the increased Hill slopes may be a reflection of desensitization, which may involve a higher degree of cooperativity than receptor activation, a hypothesis that we are continuing to investigate.

Specific Aim #3: To Determine If a Chemical Released with ACh Modulates Its Function at the Receptor/Channel Molecule

Because ATP is stored with ACh in vesicles (Whittaker & Dowdall, 1975), and is most likely released with it at motor nerve terminals, it was of interest to determine if ATP affects ACh-receptor binding. Initial assays showed that ATP did not affect K_D or B_{max} of [^{3}H]ACh binding. Since ATP is a substrate for a protein kinase which is present in the postsynaptic membrane, and some of the receptor subunits go through phosphorylation and dephosphorylation (Gordon et al., 1977), we tested the effect of ATP and other phosphorylated nucleotides on the binding of [^{3}H]H_{12}-HTX which detects conformational dependent effects. There was no effect on either resting or agonist-activated receptors (Table 4).

Table 4. Effect of phosphonucleotides on the binding of 2 x 10^{-9} M [^{3}H]H_{12}-HTX to resting and activated ACh-receptors in Torpedo membranes

Nucleotide	Binding of [^{3}H]H_{12}-HTX % of control	
	Resting receptors	Activated receptors
Guanidine HCl	96 ± 8	99 ± 4
Adenine SO_4	94 ± 7	102 ± 1
Guanosine	128 ± 12	106 ± 2
Guanosine monophosphate	93 ± 6	104 ± 2
Guanosine diphosphate	140 ± 7	106 ± 1
Guanosine triphosphate	117 ± 3	114 ± 1
Adenosine triphosphate	106 ± 2	101 ± 1
Adenylimido diphosphate	79 ± 11	110 ± 2
Inosine triphosphate	133 ± 5	104 ± 1

Specific Aim #4: To Define the Drug Specificity of the Glutamate Receptor/ Channel System

There are two major differences in neural regulation of skeletal muscles between vertebrates and invertebrates. First, the motor neurons regulating muscle activation in vertebrates are cholinergic, whereas they are glutamergic in most invertebrates (Usherwood, 1981). Second, inhibition of motor neuron action in vertebrates is central through cholinergic inhibitory interneurons, while inhibition of the invertebrate muscle is peripheral by inhibitory GABA-ergic neurons (Cull-Candy, 1982). The peripheral location of glutamate and GABA receptors in insects has facilitated their electrophysiologic investigations and provided us with detailed information of their respective ionic channels.

We utilized Na^+-independent stereoselective [^{3}H]glutamate binding (Fig. 9) to identify a glutamate receptor in housefly muscle using techniques that were successful in identifying a mammalian brain glutamate receptor with a few modifications. We included several antiproteases during membrane preparation because the insect preparation contains high protease activities. Binding to

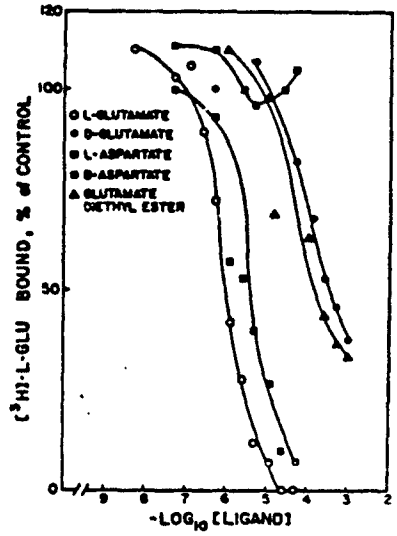

Fig. 9. Inhibition of specific [^{3}H]L-glutamate (100 nM) binding by various glutamergic ligands. Incubation was for 2 h at 4°C. Results are the means of at least 2 experiments with S.E. <8%. (From Filbin et al., in press)

the muscle membranes was saturable (Fig. 10) with a K_d of 0.5 ± 0.04 μM, which is in the same range as those reported for mammalian brain receptors (Sharif & Roberts, 1980; Michaelis et al., 1983; Baudry & Lynch, 1981). The two receptors were also similar in high sensitivity to L-glutamate and L-aspartate, but low sensitivity to quisqualate. They differ in the low sensitivity of the muscle receptor to ibotenate (Table 5). The potentiation of [^{3}H]L-glutamate binding by d-tubocurarine is most interesting because this drug was shown to block glutamate-activated open channels (Cull-Candy &

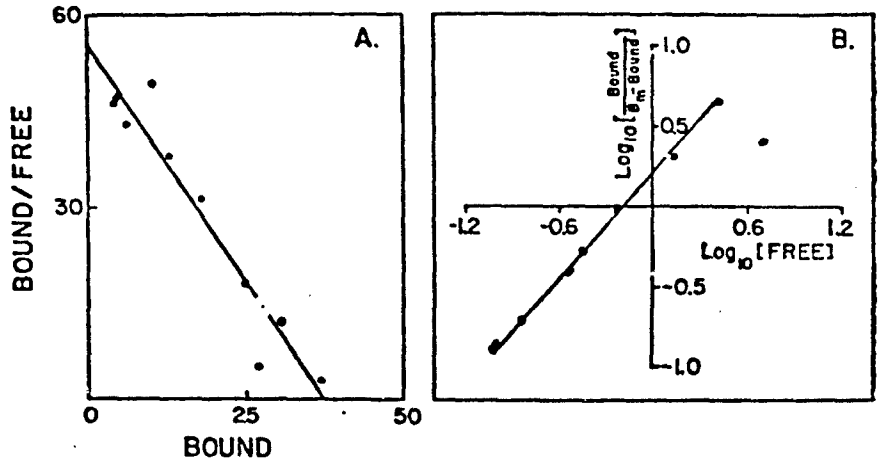

Fig. 10. Specific [^{3}H]L-glutamate binding to housefly thoracic membranes. A. Scatchard analysis of a typical experiment. Bound in pmol/mg prot. and free in μM. B. Hill plot of the same data. (From Filbin et al., in press)

Table 5. Inhibition of specific binding of 100 nM [^{3}H]L-glutamate to housefly thoracic membranes by various ligands

Ligand (1 mM)	[^{3}H]L-GLU bound % of control	Ligand (1 mM)	[^{3}H]L-GLU bound % of control
L-Glutamate	0	Proctolin	(92)
L-Aspartate	0	Kainate	(97)
L-Glutamate diethylester	7	D-Aspartate	(100)
D-Glutamate	15	GABA	(105)
N-Methyl-D-aspartate	30	Glycine	84
Ibotenate	52	Carbamylcholine	73
N-Methyl-DL-aspartate	78	d-Tubocurarine	128
Quisqualate	84	4-Aminopyridine	86
N-Methyl-L-aspartate	86	Tetraethylammonium	(105)

Results are the means of at least 2 experiments, each in triplicate (S.E. ± 10%). Values in parenthesis represent nonsignificant effect.

Miledi, 1983). By analogy with nicotinic ACh-receptor, where binding of most channel blocker increases the affinity of the nicotinic receptor for ACh, the d-tubocurarine effect is expected, and warrants further detailed investigation.

Since the venom of the Philanthus wasp was reported to be a blocker of the channel of the glutamate receptor (Clark et al., 1982), we collected this venom as detailed below and tested its effect on binding of [^{3}H]glutamate to rat brain receptors and on excitatory neuromuscular transmission in locust leg muscle. The crude venom inhibited [^{3}H]glutamate binding and transformed a

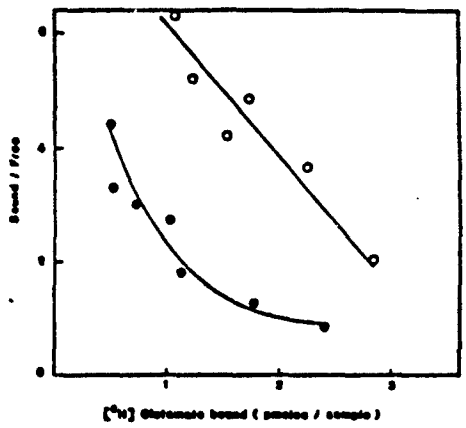

Fig. 11. Scatchard plots of specific [^{3}H]glutamate binding at 100 nM to rat brain membranes in absence (●) and presence (o) of 0.1 unit of Philanthus venom/ml.

linear Scatchard plot to a curvilinear one (Fig. 11). The data suggested that it contained a competitive inhibitor of [^{3}H]glutamate binding that may be free glutamate as well as a noncompetitive inhibitor as shown by the change in maximal binding at low venom concentrations. It also caused a voltage-dependent inhibition of epc in locust muscle, suggesting blockade of the channel of the glutamate receptor.

Specific Aim #5: To Define the Drug Specificity of the GABA Receptor/Channel System

To study the biochemical properties of GABA receptors of insect muscle we developed a binding assay that utilizes [^{3}H]flunitrazepam ([^{3}H]Flu) as an allosteric ligand-probe (Abalis et al., 1983). Initially, we found that nonspecific binding of [^{3}H]GABA to muscle plasma membrane preparations obtained from housefly thoraces was too high and variable to allow the accurate determinations of GABA receptors in insect muscles. Since benzodiazepine receptors are found usually in molecular association with GABA receptors in mammalian brain we tested for binding of [^{3}H]Flu in insect muscle. Not only did we find high affinity binding, but also the binding was increased by GABA (Fig. 12), as was found in mammalian brain, suggesting that the two receptors are similarly coupled in the insect muscles. We have used this assay to study the drug specificity of the GABA receptor/channel system and are currently investigating the use of [^{3}H]muscimol binding as well to determine if insect muscle GABA receptors are Type I or II receptors.

The drug specificity of this GABA/benzodiazepine receptor of housefly muscle is different from both the central and peripheral receptors of mammals. For example, bicuculline, which is an antagonist of the mammalian brain receptors (mostly Type I), was an agonist at low concentrations but an antagonist at higher concentrations on insect muscle receptors (Table 6). Also, the relative affinities of diazepam and clonazepam to GABA receptors of mammalian brain and insect muscle were reversed. Diazepam had 27-fold lower affinity than clonazepam for the mammalian receptor, whereas it had three orders of magnitude higher affinity for the insect receptor.

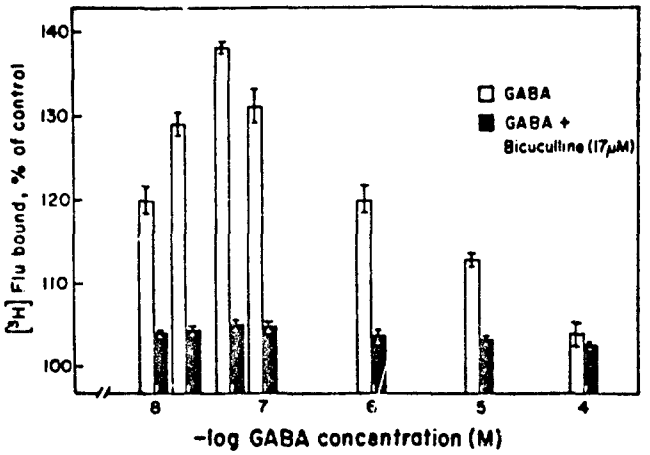

Fig. 12. The effect of GABA on the specific binding of 2.5 nM [^{3}H]Flu to membranes from housefly thorax in 5 mM Tris-HCl buffer, pH 7.1. Control 100% level represents the specific binding in absence of GABA. Open columns represent the specific [^{3}H]Flu binding in presence of the indicated concentration of GABA. Solid columns represent binding in presence of both GABA and 17 μM bicuculline. Vertical bars represent standard deviation of four experiments. (From Abalis et al., 1983)

Table 6. Comparison of benzodiazepine binding sites in different species and tissues (From Abalis et al., 1983)

Compound	K_i (nM)		
	House fly thorax[a]	Mammalian brain	Rat kidney
Flunitrazepam	290 ± 35	2.72[b]	—
Diazepam	488 ± 60	27.4[b]	—
Clonazepam	146,000 ± 3,522	1.13[b]	1.79[d]
Ro 5-3027	9,800 ± 605	1.24[b]; 4.4[c]	—
Ro 5-2180	9,800 ± 460	8.8[c]	—
Ro 5-4864	680 ± 75	100,000[d]	2.9[d]
β-CCE	9,800 ± 325	1.13[c]	—

[a] Each value is the mean of three separate experiments, performed in triplicate, ± standard deviation.
[b] Data on [^{3}H]Flu binding to human cerebral cortex membranes from Speth *et al.* (25).
[c] Data on [^{3}H]diazepam binding to rat brain membranes from Braestrup and Squires (11).
[d] Data calculated from IC_{50} values of [^{3}H]diazepam binding to rat tissues from Braestrup and Squires (39).
[e] Data calculated from IC_{50} values of [^{3}H]Flu binding to rat cerebellum from Braestrup *et al.* (25).

We discovered that several environmental toxicants affected the GABA/benzodiazepine/picrotoxinin receptor complex in housefly muscle and rat brain.

Both Type I and Type II pyrethroids (at 10^{-4} M) enhanced the specific binding of [^{3}H]Flu to GABA receptors and the presence of GABA (10^{-5} M) caused a significant additional increase (Table 7). It suggests that pyrethroids bind to the benzodiazepine/GABA complex at an allosteric site and affect [^{3}H]-Flu binding. Their action is also affected by GABA through allosteric intramolecular transitions. This viewpoint supports the recent finding that pyrethroids bind to the picrotoxinin binding component of the GABA receptor complex (Lawrence & Casida, 1983). However, only Type II pyrethroids had this effect.

Recently, avermectin, a microcyclic lactone derived from the Streptomyces avermilitis, was shown to be a potent poison for invertebrates (e.g., insects and worm parasites), and it was suggested that its action was directed against GABA transmission, possibly through modulation of GABA receptor (Supavilai & Karobath, 1981). Avermectin at concentrations >μM interacted with the GABA receptors of rat brain and insect muscle and caused significant and dose-dependent increases in [^{3}H]Flu binding to both receptors (Table 8). Furthermore,

Table 7. The effect of pyrethroids on the binding of [^{3}H]Flu to rat brain membrane

Pyrethroids (100 μM)	Specific [^{3}H]Flu binding (fmole/mg protein)[a]	
	- GABA	+ 10^{-5} M GABA
Control	136 ± 22	225 ± 33
Type I		
Pyrethrins	152 ± 24	241 ± 11
Resmethrin	132 ± 20	261 ± 20
Allethrin	167 ± 15	257 ± 25
Tetramethrin	200 ± 30	292 ± 34
Bioallethrin	163 ± 20	233 ± 22
Type II		
Permethrin	132 ± 25	233 ± 15
Fenvalerate	180 ± 32	300 ± 31
Fluvalinate	175 ± 34	280 ± 27
Cypermethrin	163 ± 16	263 ± 16
BAY FCR 1272.127	200 ± 38	296 ± 40

[a]Values are presented as means ± standard deviations of triplicate determinations, [^{3}H]Flu 0.85 nM.

Table 8. Effect of avermectin on the binding of 10^{-9} M [^{3}H]Flu to housefly muscle and rat brain membranes in presence and absence of 10^{-6} M GABA

[Avermectin] (M)	Binding of [^{3}H]Flu as % of control			
	Housefly muscle		Rat brain	
	-GABA	+10^{-7} M GABA	-GABA	+10^{-6} M GABA
0	100	130	100	154
10^{-9}	---	---	109	160
10^{-8}	108	127	124	170
10^{-7}	97	141	142	185
10^{-6}	93	152	157	207
10^{-5}	131	178	192	220
10^{-4}	273	195	239	263

the presence of GABA caused additional increases. Again these results suggest that avermectin is binding to an allosteric site to both the GABA and benzodiazepine binding sites. It may be the same site where picrotoxin and pyrethroids bind.

Another group of environmental toxicants that had been suggested to interact with the GABA receptor complex is the cyclodiene insecticides. Several cyclodienes and γ-hexachlorocyclohexane were tested for their effects on the binding of [^{3}H]Flu in presence and absence of GABA to rat brain GABA receptors (Fig. 13). In all cases, the cyclodienes caused significant increases in [^{3}H]Flu binding, and GABA caused additive increase, effects that are similar to those produced by pyrethroids and avermectin.

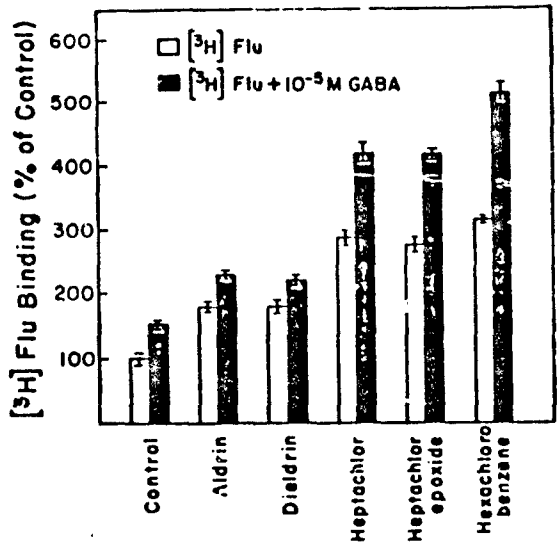

Fig. 13. Histogram of the effect of 10 μM cyclodienes on [^{3}H]Flu binding at 0.5 nM to rat brain membranes in presence and absence of 10 μM GABA.

Preliminary experiments with crude venom extracts from Philanthus wasp showed that the venom increased [^{3}H]Flu binding to rat brain and housefly muscle receptors. This increase was partially due to free GABA bound in the venom extract. However, another factor of much larger molecular weight, isolated by chromatography on Sephadex G-25, produced the same effect. Further investigations with pure venom components are needed.

Specific Aim #6: To Study the Interactions of Anticholinesterases with the ACh-, Glutamate- and GABA-Receptor/Channel

Several anticholinesterases interacted with the ACh-receptor. We investigated the structure-activity relationship of 16 quaternary ammonium anticholinesterases on the activity of ACh-esterase and on the binding of [^{3}H]-ACh and [^{3}H]PCP to the "receptor" and "channel" sites, respectively, and on the receptor-regulated $^{22}Na^+$ influx into Torpedo microsacs (Bakry et al., 1982). The study showed that the structural requirements for inhibiting ACh-esterase were more rigid than for binding to the receptor sites, and there was no correlation between their potencies on the enzyme and receptor. Introduction of an m-hydroxy group on the phenyltrimethylammonium had little effect on binding to the receptor site, but dramatically increased its anti-ACh-esterase potency. Most ammonium anticholinesterases studied activated the nicotinic receptor like agonists, though with low potency. Some quaternary ammonium compounds also bound to the allosteric channel sites and displaced binding of the allosteric probe [^{3}H]PCP. Increasing the chain length of a homologous series of symmetrically substituted tetraalkylammonium compounds from tetramethyl to tetrahexyl decreased affinity for the receptor sites and increased it for the channel sites (Fig. 14). Edrophonium had equal affinity for ACh-esterase and ACh-receptors. It bound to the receptor sites and activated the receptor like an agonist, but at higher concentrations it bound to the ionic channel sites; thus it acted like decamethonium.

Neostigmine (Neo), pyridostigmine (Pyr) and physostigmine (Phy) at low concentrations (<μM) inhibited ACh-esterase, thereby indirectly potentiating ACh enhancement of [^{3}H]H_{12}-HTX binding to the channel sites of the nicotinic ACh-receptor of Torpedo membranes. However, at higher concentrations they inhibited ACh action due to direct interactions with the ACh-receptor. The three carbamates displaced binding of [^{3}H]ACh to the receptor sites in membranes whose ACh-esterase was totally inhibited (Fig. 15A), and also potentiated binding of [^{3}H]H_{12}-HTX (Fig. 15B). Thus, they acted as weak agonists with the following rank order of potency: Neo > Pyr > Phy. On the closed receptor channel (i.e., in membranes pretreated with Naja α-neurotoxin) only Phy above 100 μM inhibited [^{3}H]H_{12}-HTX binding (Fig. 16). However, on the carbamylcholine-activated receptor channel, the three carbamates inhibited [^{3}H]-H_{12}-HTX binding with Phy as the most potent. The potency of Phy was not due to the lack of positive charge since Phy methiodide acted similarly. Preincubation of ACh-receptors with the carbamates reduced the rate of binding of [^{125}I]α-BGT and increased the rate of carbamylcholine inhibition of [^{125}I]α-BGT binding (Fig. 17) with the following order of potency: Neo > Phy > Pyr, suggesting that they induce and potentiate receptor desensitization. In summary, the three carbamates interact with the nicotinic ACh-receptor, with Neo and Pyr acting mainly as weak agonists and Phy as an open channel blocker.

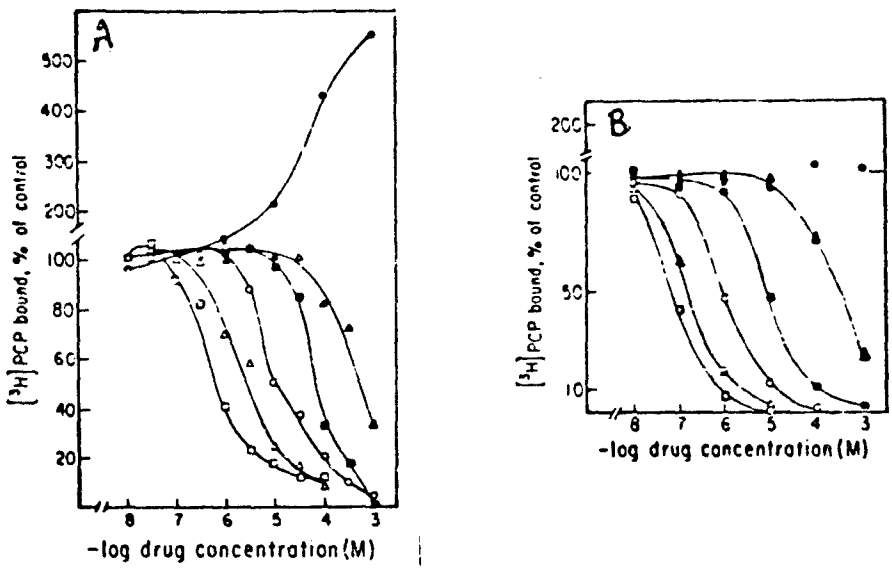

Fig. 14. Log dose-response relationships of the effect of six symmetrically substituted tetraalkylammonium compounds on the binding of [^{3}H]PCP (2 nM) to the channel sites of the ACh-receptor in Torpedo membranes, in absence (A) or presence (B) of 100 μM carbamylcholine measured after 30 sec. The drugs used were tetramethylammonium (●), tetraethylammonium (▲), tetrapropylammonium (■), tetrabutylammonium (o), tetrapentylammonium (Δ), and tetrahexylammonium (□). Incubation time was 30 sec, and nonspecific binding (i.e., binding in presence of 5 mM amantadine) was subtracted. Symbols represent means of three experiments; the standard deviation was <10%. (From Bakry et al., 1982)

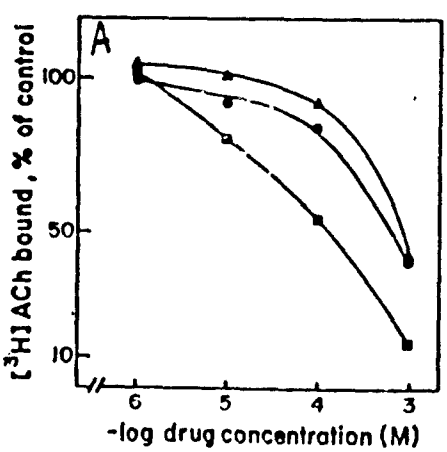

Fig. 15A. Inhibition of the binding of [^{3}H]ACh to Torpedo ACh-receptors by carbamate anticholinesterases: Phy (●), Pyr (▲) and Neo (■). Membranes were preexposed to 100 μM DFP for 30 min.

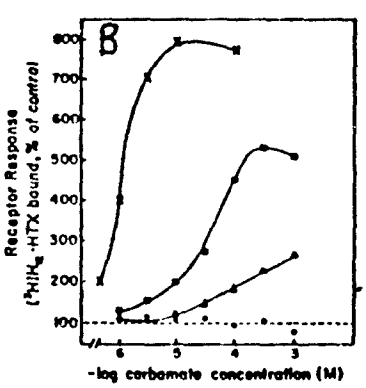

Fig. 15B. The effects of carbamylcholine (x), Neo (■), Pyr (▲), and Phy (●) on binding of [^{3}H]H_{12}-HTX to nicotinic ACh-receptor in Torpedo membranes. The dashed line represents control level binding in absence of drugs.

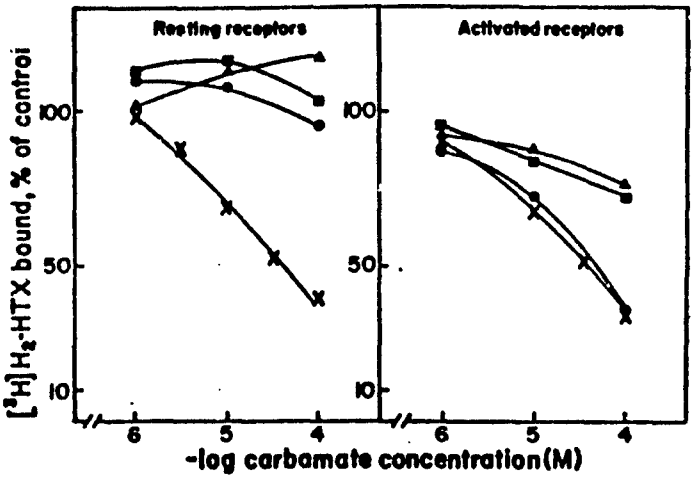

Fig. 16. The effects of amantadine (x) (a channel blocker that displaces $[^3H]H_{12}$-HTX binding), Phy (●), Pyr (▲) and Neo (■) on the binding of 2 nM $[^3H]H_{12}$-HTX to the resting ACh-receptors (where the membranes were pretreated with Naja α-neurotoxin (10 μM) measured after 2 h) and activated ACh-receptors (where membranes were incubated with 100 μM carbamylcholine, drug and $[^3H]H_{12}$-HTX) and binding was measured after 30 sec. Amount of $[^3H]H_{12}$-HTX bound by control resting receptors at equilibrium was similar to that bound by control carbamylcholine-activated receptors in 30 sec.

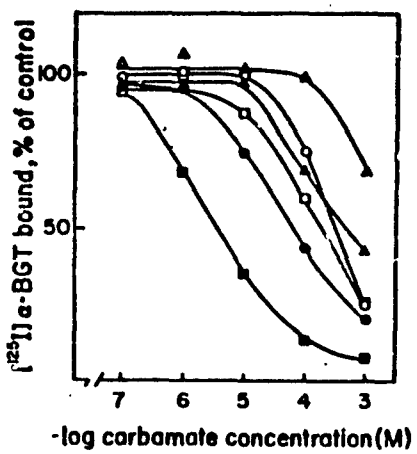

Fig. 17. Inhibition of the binding of $[^{125}I]$α-BGT to Torpedo ACh-receptors by Phy (o,●), Pyr (Δ,▲) and Neo (□,■). Membranes were exposed to drugs either simultaneously with $[^{125}I]$α-BGT for 40 sec (open symbols) or preincubated with the drug for 30 min before exposure to $[^{125}I]$α-BGT for 40 sec (solid symbols). Preincubation increased the affinity of the ACh-receptors for the three carbamates.

Effects of Anticholinesterases on Glutamate and GABA Receptors

The anticholinesterases tested had no effect on glutamate and GABA receptors in rat brain (Table 9). However, one should bear in mind that they were tested only on binding of two radiolabeled ligands, and they may affect binding of other ligands to these receptor systems such as picrotoxinin binding to the GABA receptor channel, or [^{3}H]aminophosphonobutyrate to a Ca^{2+}-Cl^- sensitive subtype of glutamate receptors.

Table 9. Effect of anticholinesterases on the binding of [^{3}H]Flu (10^{-9} M) and [^{3}H]glutamate (10^{-7} M) to rat brain receptors

Drug (10^{-4} M)[a]	Binding as % of control		
	[^{3}H]Flu		[^{3}H]Glutamate
	- GABA	+ 10^{-6} M GABA	
Physostigmine	104 ± 3	101 ± 5	
Neostigmine	103 ± 2	99 ± 4	--
Pyridostigmine	100 ± 3	98 ± 2	--
Edrophonium	97 ± 5	101 ± 3	100 ± 1
Tetraethylammonium	102 ± 6	99 ± 1	

[a]All drugs were also tested at 10^{-8} and 10^{-6} M, but no significant differences from control levels were observed.

Collection of Philanthus Venom and Testing

Support was provided through a supplement from ARO to collect Philanthus wasp, whose venom was reported to contain an inhibitor of glutamate receptors. It had previously been collected from Egypt. Therefore, the first step was to determine its densities in different areas there. It was found in highest concentrations in the Dakhla oasis where about 5,000 wasps were collected over a period of three months, the glands and venom sacs were removed, frozen in liquid nitrogen then lyophilized.

This honey bee wolf (Philanthus) uses a paralytic venom to immobilize honey bee workers and steal their honey. Water extracts of lyophilized venom sacs and glands injected into honey bee workers produce immediate paralysis, with recovery that is dependent on the injected dose. One-tenth of a venom unit (i.e., contents of a venom sac and glands of a single wasp) produces paralysis that lasts for 30 min. Preliminary results of Sephadex gel chromatography (Fig. 18) and polyacrylamide gel electrophoresis (Fig. 19) indicate that the crude venom has multiple components including amino acids, in particular GABA and glutamate, and several polypeptides.

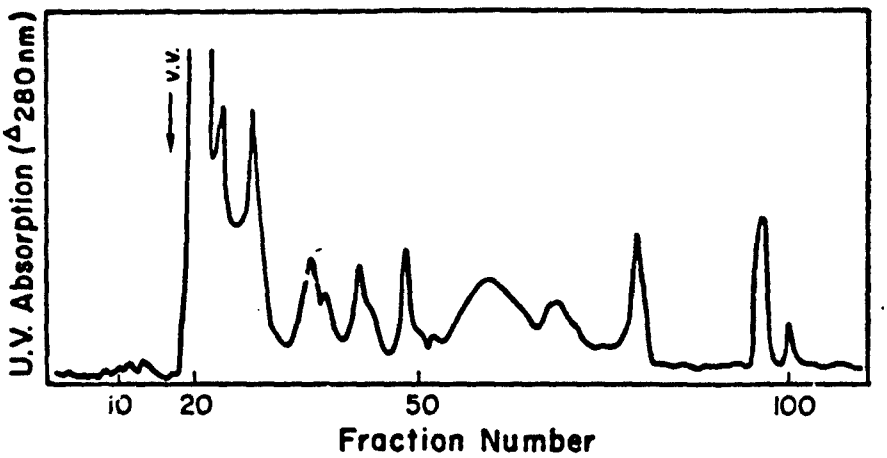

Fig. 18. Chromatographic separation of the components of Philanthus venom on Sephadex G-25 using distilled water for elution. Fraction volume was 1 ml, and flow rate was 40 ml per hour. Column dimension was 1 cm diameter and 90 cm height. (Courtesy of Dr. Clarence Broomfield)

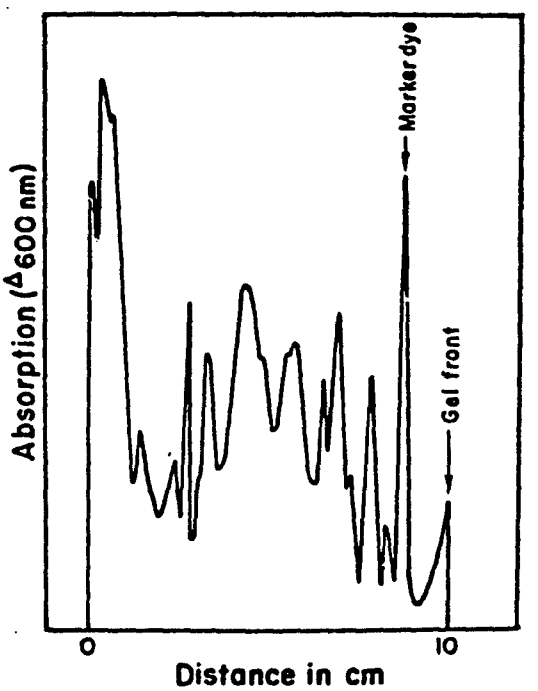

Fig. 19. Electrophoretic separation of the components of Philanthus venom by disc gel electrophoresis using 7% separating gel, pH 9.0 (nondenaturing conditions). Gels were stained with coomassie blue and scanned on a Beckman spectrophotometer. Gel length was 10 cm and bromophenyl blue was used as a marker dye.

Effects of crude venom extracts on ligand binding to nicotinic ACh-receptors of Torpedo electric organs and insect ganglia indicate that the venom contains more than one active component and has no anticholinesterase activity. It inhibits [^{125}I]α-BGT binding to the ACh-receptor, and preincubation of receptors with the venom increases receptor affinity for agonists due to desensitization. The venom also increases the initial rate of binding of [^{3}H]H_{12}-HTX to the ACh-receptor channel, which is due to receptor activation. These results suggest that the venom has a component which acts like an agonist on nicotinic ACh-receptor. This component is acid stable, insensitive to proteases and ACh-esterase, but is sensitive to alkaline pH. The venom also contains a noncompetitive antagonist of the nicotinic ACh-receptor, which blocks binding of [^{3}H]H_{12}-HTX to the channel sites of the agonist-activated receptor.

The crude venom extract proved also to contain a noncompetitive inhibitor of glutamate receptor as detected by its effect on endplate current and specific binding of [^{3}H]glutamate (see above).

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Abstracts

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D. LIST OF PARTICIPATING PERSONNEL

Professional Personnel

Name and Title	Employment Dates	% Effort
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Dr. Edson X. Albuquerque, Co-principal Investigator	9/28/81-9/27/84	20
Dr. Amira T. Eldefrawi, Res. Professor	9/28/81-9/27/84	30
Dr. Esam F El-Fakahany, Res. Associate	9/28/81-6/30/83	100
Dr. Geoffrey Schofield, Res. Associate	9/28/81-10/31/81	100
Dr. Marie T. Filbin, Res. Associate	3/1/82-5/15/82	100
Dr. Mohammed A. Maleque, Res. Asst. Prof.	7/1/82-11/30/82	100
Dr. Wamberto A. Varanda, Res. Associate	2/27/83-9/30/83	100
Dr. Charles N. Allen, Res. Associate	6/1/83-6/30/83	100
Dr. Shebl M. Sherby, Res. Associate	8/1/83-9/30/83	100
" " " "	2/24/84-9/27/84	100
Dr. K. S. R. Rao, Res. Associate	12/31/83-9/27/84	100
Mr. Luis G. Aguayo, Graduate Student	12/31/83-9/27/84	100

None earned advanced degrees while employed by project.

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